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The

Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 15 *November 1914* No. 3

THE METABOLISM OF SAPROPHYTIC HUMAN TUBERCLE BACILLI IN PLAIN, DEXTROSE, MANNITE, AND GLYCERIN BROTHS

STUDIES IN ACID-FAST BACTERIA. I*

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One of the noteworthy contributions to the chemistry of the tubercle bacillus was the discovery by Theobald Smith[1] that human tubercle bacilli produce a terminal acid reaction in nutrient glycerin broth, contrasting in this respect with bovine tubercle bacilli, cultures of which tend to an alkaline reaction in the same media. Attention was directed by him to slight variations in this reaction of the human bacilli; if the medium is not favorable for the luxuriant growth of these organisms, this terminal acidity may be lessened or even be absent. Furthermore, as he pointed out, this acid reaction is developed only when the glycerin content of the broth is 1 percent or more; a concentration of at least 2 percent of glycerin is necessary for the maximum acidity. Again, this acidity is a differential acidity, for alkaline products appear to be formed in glycerin broth coincidently with the

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* Received for publication July 27, 1914.

[1] Trans. Assn. Am. Phys., 1903, 18, p. 108; Jour Med. Research, 1905, 13, p. 253; Am. Jour. Med. Sci., 1904, 128, p. 216.

acid products derived from the glycerin. A differential excess of acid, rather than a purely fermentative decomposition of glycerin, is responsible for this noteworthy difference between the human and bovine types of tubercle bacillus. The latter organism does not appear to form acid from glycerin. The development of alkalinity, probably derived from protein constituents of the broth, is brought out sharply when bovine and human bacilli, respectively, are grown in broth cultures containing no glycerin. In this medium, altho the growth is less luxuriant than in glycerin broth, the reaction becomes progressively alkaline in both instances.

This work is important from another point of view. Prior to the publication of these studies, investigators, almost without exception, assumed that tubercle bacilli could not be cultivated in artificial media unless glycerin was present. Some observers have gone so far as to state dogmatically that glycerin was a *sine qua non* for the artificial cultivation of these organisms. The observations mentioned above show definitely that the majority of strains of tubercle bacilli will develop, altho much less luxuriantly, even in plain nutrient broth. These organisms, therefore, are not obligately glycerophilic.

Dextrose, or muscle sugar, appears to play no part whatsoever in the development of tubercle bacilli in glycerin broth, for the amount of dextrose remains the same in broth after prolonged cultivation of these organisms in it as in uninoculated broth, as shown by the fermentation test with the colon bacillus. These observations were soon confirmed, and they were apparently accepted without question by Koch as the only practical cultural differentiation between human and bovine bacilli.

From time to time strains of tubercle bacilli have been isolated from man which appear to be intermediate between the human and bovine types when judged by this criterion, but for the most part subsequent investigation has shown that these few intermediate strains revert, after prolonged cultivation, to one type or the other, or they have been definitely referred to one of the other types by careful animal experiments. A few strains, however, appear to be exceptional in this respect.

The experiments here recorded were undertaken with the specific purpose of studying the gross metabolism of tubercle bacilli in artificial media, particularly with reference to the influence of glycerin on the metabolism of these organisms.

The organisms studied in this connection were two strains of rapidly growing human tubercle bacilli; one of these, "W", was kindly sent us by Professor Wherry of the University of Cincinnati; the other, "597", from the Museum of Natural History, New York, by Professor Winslow. Culture "W" is a descendant of a culture from Koch's laboratory, brought to this country by Professor Vaughan of the University of Michigan. It is avirulent for guinea-pigs. Culture "597" will produce tuberculosis in guinea-pigs if relatively large doses are injected intraperitoneally. It should be emphasized that these cultures, particularly "W", in virtue of their avirulence for guinea-pigs, might be questioned with respect to their identity as human tubercle bacilli.

Further observations along similar lines with human bacilli of known virulence, however, which will be presented later, have given reactions qualitatively identical with those mentioned above, and the study of a considerable series of acid-fast bacilli, not tubercle bacilli, have shown unmistakable cultural differences which appear to separate them from these avirulent tubercle bacilli.

Rapidly growing tubercle bacilli offer advantages for chemical study which are obvious; their relatively active development leads to an early accumulation of decomposition products in measurable amounts, and these products are less influenced by parallel recessive changes due to autolysis of the bacilli themselves than appears to be the case with the highly virulent, slowly growing strains.

The media selected for this study comprise nutrient sugar-free broth made from meat juice as a basis. Portions of this broth were re-enforced by 1 percent dextrose, 1 percent mannite, and 3 percent of glycerin, respectively, as additional sources of carbon. As in previous experiments,[2] several flasks of the same size and shape [250 c.c. Erlenmeyer flasks], each containing 100 c.c. of the medium, were prepared at the same time and inoculated and incubated under similar conditions of temperature and moisture. The methods of analysis used have also been described in detail elsewhere[3] and need not be referred to here. The determinations comprise: changes in reaction, using alizarin, neutral red, and phenolphthalein as indicators; and the production of ammonia and a parallel study of the morphology, using the Ziehl-Neelsen stain. The stains for a given series were all made at the same time and subjected to the same degree of heat, decolorization, etc. to obviate errors in this direction as far as possible. The results based on the initial volume of 100 c.c. of media follow, the reaction being expressed in terms of cubic centimeters of normal acid or alkali per hundred cubic centimeters of broth, the ammonia as milligrams of nitrogen per 10 c.c. of broth. Several parallel experiments, some in duplicate, were made at different times in different lots of media. These are included to indicate the approximate limits of variation which may be expected.

The only noteworthy features in the morphology and staining reactions were a change about the end of the fourth week from the typical, rather long, slender rod to a short, thick rod, and a moderate proportion of non-acid-fast rods, typical, however, morphologically during the first two weeks of growth. These short, thick rods resemble strikingly the bovine bacillus. The experiments, however, do not suggest that a further cultivation of these short rods would result in a permanent change in morphology from organisms resembling the human type of the bovine bacillus to that of the bovine. The change from partial acid-fastness to complete acid-fastness seems to be somewhat closely associated with a recession in chemical activity. Wolbach and Ernst[4] have shown that young cultures of tubercle bacilli exhibit a certain degree of non-acid-fastness. Neither dextrose, mannite, nor glycerin spare protein to any such degree as dextrose, for example, spares protein under similar conditions with a great majority of the commonly met with bacteria.

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[2] Kendall, Day and Walker, Jour. Am. Chem. Soc., 1912, 35, p. 14.

[3] Kendall and Farmer, Jour. Biol. Chem., 1912, 12, p. 13.

TABLE 1
METABOLISM OF TUBERCLE BACILLI IN BROTH

Bacillus tuberculosis	Days	Plain Broth					Dextrose Broth				
		Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent	Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent
597	4	—1.10	0.00	—0.20	5.6	2.50	0.00	0.00	—0.20	4.9	2.50
	6	—2.50	—0.50	—0.50	10.5	4.69	—0.20	0.00	—0.10	5.6	2.85
	13	—3.40	—1.20	—0.90	26.6	11.87	—1.80	—0.50	—0.10	23.1	11.80
	20	—3.00	—1.40	—1.10	21.0	9.37	—3.20	—1.50	—0.80	30.8	15.70
	27	—3.30	—1.50	—2.00	15.4	6.88	—2.60	—1.70	—1.00	21.7	11.60
	34	—2.10	—1.50	—0.90	16.1	7.23	—1.70	—1.20	—0.90	15.4	7.85
W	4	—1.10	0.00	—0.30	2.8	1.25	—0.20	0.00	—0.20	4.9	2.50
	6	—2.30	—0.40	—0.50	8.4	3.75	—0.20	0.00	—0.20	4.9	2.50
	13	—2.80	—1.10	—0.80	25.2	11.25	—2.70	—1.20	—0.50	23.1	11.80
	20	—3.20	—1.50	—0.90	26.6	11.88	—2.80	—1.50	—0.80	23.1	11.80
	27	—2.90	—1.40	—0.60	23.8	10.63	—2.10	—1.30	—0.80	24.5	12.50
	34	—1.10	—1.50	—0.90	16.8	7.50					
							—1.40	—1.20	—0.60	11.2	5.72
W1	1	—0.30	0.00	—0.20	2.1	0.68	—0.30	—0.10	0.00	2.1	0.68
	4	—1.60	—0.90	—0.40	5.6	1.81	—1.50	—0.60	—0.20	1.4	0.45
	7	—3.30	—1.40	—1.50	22.4	7.30	—3.40	—1.40	—0.70	23.1	7.50
	14	—3.60	—2.00	—1.40	26.6	8.62	—3.70	—2.20	—1.10	24.5	7.98
	21	—3.60	—2.30	—1.30	24.5	7.98	—3.00	—2.20	—0.90	23.8	7.72
	28	—3.20	—2.20	—1.30	14.7	4.77	—3.00	—2.40	—1.40	23.8	7.72
	35	—2.90	—1.40	—1.30	8.4	2.79	—2.60	—1.60	—1.60	7.0	2.33
W2	1	0.00	+0.10	0.00	0.70	0.24	—0.20	—0.20	—0.10	0.70	0.23
	4	—1.10	—0.50	—0.30	4.2	1.46	—1.60	—0.90	—0.50	5.6	1.86
	7	—2.70	—1.40	—1.10	17.5	6.10	—3.00	—1.40	—0.80	21.0	6.98
	14	—2.90	—2.00	—1.20	18.2	6.35	—3.60	—2.60	—1.10	30.8	10.70
	21	—2.80	—1.60	—1.20	14.7	5.13	—3.10	—2.00	—1.60	14.0	4.65
	28	—2.60	—1.50	—1.30	9.1	3.17	—2.60	—2.20	—1.70	9.1	3.02

Throughout these experiments the growth in plain broth was distinctly less luxuriant and less extensive than that occurring in dextrose, mannite, or glycerin media. The growths in the later media were of about equal magnitude. After a period of alkalinity the reaction gradually becomes acid in glycerin broth, and progressively alkaline in plain, dextrose, and mannite broths. This is in accord with Theobald Smith's observations mentioned above. The changes in ammonia production would indicate that this development of acid is not to be interpreted as a sparing action of the glycerin for protein. Perhaps the most striking feature of the metabolism is the rather general recession of the ammonia. Ammonia production reaches its maximum about the second or third week of incubation, and then gradually diminishes, so that the final amount of ammonia detectable in the cultures is usually much less than that observed at the height of vegetative activity. This phenomenon has not been observed in cultures of other bacteria which have been studied similarly. The reason for this recession is not apparent. Several possible explanations present themselves. Loss of ammonia by volatilization is not very probable, for the solution is not unduly alkaline and the small amount of ammonia formed, taken into con-

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[4] Jour. Med. Research, 1903, 10, p. 313.

TABLE 1—(*Continued*)

METABOLISM OF TUBERCLE BACILLI IN BROTH

Bacillus tuber-culosis	Days	Mannite Broth					Glycerin Broth				
		Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent
597	4	—0.40	+0.10	—0.10	—2.1	—0.97	+0.50	+0.30	+0.20	0.00	0.00
		—0.30	+0.10	—0.10	—2.1	—0.97	—0.50	+0.10	+0.10	6.3	3.10
	13	—1.90	—0.60	+0.20	21.7	10.00	—1.00	—0.10	+0.40	24.5	12.05
	20	—2.10	—0.90	—0.40	35.2	14.85	—1.30	—0.30	+0.60	35.0	17.25
	27	—2.60	—1.20	—0.60	29.4	15.55	—1.00	—1.10	+0.40	39.9	19.65
	34	—2.00	—1.40	—0.20	29.4	15.55	—0.80	—0.10	+1.20	44.1	21.70
W	4	—0.50	+0.20	—0.10	0.00	0.00	—0.80	+0.10	—0.30	2.1	1.03
	6	—0.70	+0.10	0.00	4.2	1.93	—0.30	+0.20	0.00	2.1	1.03
	13	—3.00	—1.30	—0.80	34.3	15.80	0.00	+0.30	+0.50	19.6	9.68
	20	—2.70	—1.50	—1.00	25.9	11.92	—0.50	+0.20	+0.80	27.3	13.45
	27	—2.30	—1.50	—0.90	21.7	10.00	—1.90	—0.10	+0.40	35.7	17.60
	34	—1.60	—1.20	—1.00	13.3	6.13	—0.90	—0.30	+0.40	35.7	17.60
W1	1	—0.30	—0.30	—0.20	1.4	0.47	—0.20	0.00	—0.30	1.4	0.47
	4	—1.30	—0.40	—0.50	2.1	0.70	—1.50	—0.60	—0.30	5.6	1.86
	7	—2.40	—0.80	—0.40	17.5	5.81	—2.20	—0.80	—0.30	16.8	5.58
	14	—3.70	—2.10	—1.20	35.7	11.87	—2.30	—1.20	—0.50	23.8	7.90
	21	—3.30	—3.00	—0.90	34.3	11.40	—2.30	—1.60	+0.10	26.6	8.85
	28	—3.10	—3.20	—1.60	26.6	8.82	—2.70	—1.70	+0.40	27.3	9.08
	35	—3.30	—1.50	—0.90	28.7	9.51	—2.30	—1.30	+0.70	7.7	2.55
W2	1	—0.30	—0.20	0.00	0.00	0.00	—0.10	—0.10	+0.10	—0.70	—0.24
	4	—2.90	—1.40	0.00	7.7	2.62	—1.20	—0.60	—0.10	9.1	3.17
	7	—3.40	—2.20	—1.10	23.8	8.1	—1.30	—1.10	—0.10	18.2	6.34
	14	—4.20	—2.70	—1.60	17.5	5.95	—2.40	—1.90	+0.70	32.2	11.2
	21	—2.70	—2.00	—1.50	14.0	4.76	—1.40	—1.20	+0.10	21.0	7.3
	28	—2.10	—2.10	—1.60	13.3	4.53	—1.80	—1.30	+0.20	21.7	7.53

sideration with the great solubility of this substance in water, would seem to eliminate this possibility. With our present-day knowledge of autolysis of bacterial cells it would appear that the ammonia should increase rather than diminish if this were the chief cause. In this connection it should be stated that the pellicles showed no visible diminution in size, but this does not exclude the possibility that the contents of the bacteria may have become soluble, leaving their skeletons, as it were, intact.

The fundamental composition of media made from meat juice and peptone must be extremely complex, and it is conceivable that certain substances, perhaps of the nature of fats, lipoids, or their derivatives, might play a part. These substances are present undoubtedly in small amounts in ordinary media. Tubercle bacilli contain considerable amounts of fats and their derivatives in their bodies, and it is conceivable that these substances play some part in this reaction.

CONCLUSIONS

Young, rapidly-growing tubercle bacilli appear to be, in part at least, non-acid-fast.

The strain of avirulent tubercle bacilli studied here exhibit the Theobald Smith reaction characteristic of the growth of human tubercle bacilli in glycerin broth.

Neither dextrose, mannite, nor glycerin appears to exert any marked sparing action for the protein constituents of ordinary media.

Ammonia accumulates rather rapidly during the first, second, and third weeks of growth of tubercle bacilli in plain, dextrose, mannite, and glycerin broths, followed by a definite well-marked recession, during which this ammonia detectable in the media gradually diminishes in amount. The cause of this recession is unknown.